

Disinfection of Water Systems in Accordance with Eu Directives on Health and Safety in Working Environments: Use of A Technologically Advanced ClO₂ Generator

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Abbreviations: ECDC: European Centre for Disease Prevention and Control; EU: European Union; UFC: Colony Forming Units; MPN: Most Probable Number; UG: Genomic Units

Abstract

It is currently of great interest to develop prevention systems for the management of the risk of microbial contamination of water in buildings of various types in which work is done. In this regard, international organizations have drawn up reference guidelines and in the EU countries specific legislative provisions indicated in the EU directives must be observed. A pilot system, copying a building pipeline, was designed, and developed to test water treatments with a ClO₂ generator. Chemical and physical parameters were monitored in real time and the effectiveness of disinfection was tested on several microorganisms, focusing on Legionella. The scale plant reached steady state within 24 hours and maintained equilibrium, showing stability to chemical and physical parameters. Simulations showed disinfection efficacy for different contaminations (>1,000,000 CFU) under water system conditions. *Escherichia coli* counts and other indicators showed a 99.999% reduction within 15 minutes while Legionella and other environmental species showed a 99.999% reduction within 30 minutes. In addition, the system proved to be effective on complex microflora and other species and allowed to demonstrate adherence to the regulatory framework and legislative requirements (italian legislation's accepte of EU directives on health and safety at work D.lgs. [1]. And with particular reference to the European Directive [2] and European Parliament's Directive [3].

The study model can be easily extended to simulate particular risk situations, verifying for each of them the effective compliance with current legislation. This pilot model can be a feasible support in validating or comparing alternative solutions and new technologies for disinfection of water.

Introduction

One of the main problems in the management of water in buildings is the risk of legionnaires' disease for users and workers. In fact, legionellosis is currently considered a global problem. The WHO points out those 10-15 cases/1,000,000 inhabitants are reported each year in Europe, Australia and the USA (www.who.int/en/news-room/fact-sheets/detail/legionellosis December 2016).

The bacterium responsible is Legionella pneumophila. A chlorine dioxide generator is used in the pilot water treatment plant (Figure 1). To test the effectiveness of the technologically advanced LOTUS AIR [4-20] generator a pilot system has been used. The use of chlorine dioxide in water disinfection has been shown to be more effective than chlorine against bacteria, algae, fungi, and viruses. Its

action is not aggressive against pipes and has a longer lasting effect and at a wider range from the injection point, making it possible to treat entire hydraulic systems even using small quantities of product. There are also no potentially carcinogenic disinfection byproducts, unlike chlorine, and for all these reasons its use is ecological and also suitable for drinking water treatment. As well as to combat legionella in hospitals, retirement homes, hotels, and school buildings. According to data provided by the European Centre for Disease Prevention and Control (ECDC) in 2017, between the countries of the European Union (EU) and the European Economic Area (EEA), 9238 cases of legionella were reported, with a notification rate of 1.8 per 100 thousand inhabitants, an increase compared to previous years (in 2013 it was 1.2 per 100 thousand inhabitants).

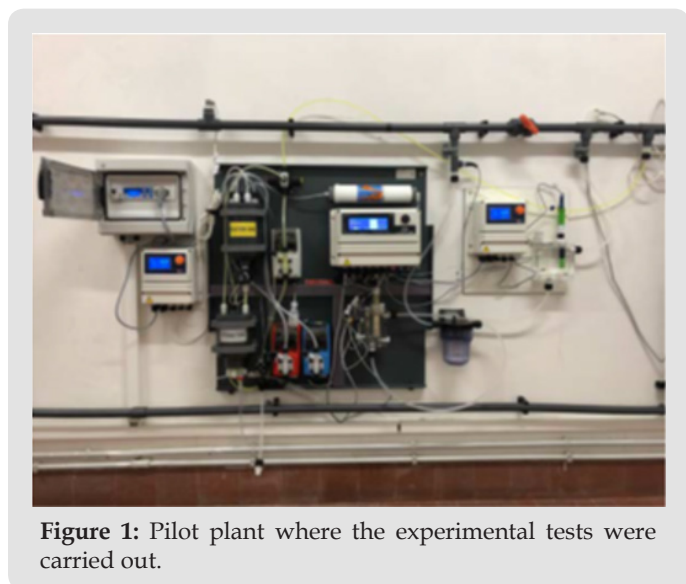


Figure 1: Pilot plant where the experimental tests were carried out.

France, Germany, Italy, and Spain together recorded 68% of cases notified in 2017. The majority of cases (69%) are of EU origin, 21% are associated with travel and 8% are of nosocomial origin. 91% of cases are over 45 years of age and the male-to-female ratio is 2.4 to 1. In addition, the lethality rate in 2017 was 8%, a value comparable to that of previous years. Laboratory analysis identified *L. pneumophila* serogroup 1 as the most frequent aetiological agent (present in 79% of cases confirmed in culture). <https://www.epicentro.iss.it/legionellosi/epidemiologia-europa> The incidence of legionellosis in Italy in 2017 was 33.2 cases per million inhabitants, a slight increase compared to last year (25.1/1,000,000), as was the absolute number of cases. However, there is a North-South gradient with values equal to 50.1 cases per million in the North, 35.1 per million in the Centre and 9.7 per million in the South [21-32]. In 2017 there were 124 (6.2 of the total number of reported nosocomial cases), of which 50 (40.3%) of confirmed nosocomial origin and 74 (59.7%) of probable nosocomial origin. Of the nosocomial cases, 73% were notified by Lombardia, Emilia-Romagna, Lazio, Provincia Autonoma di Trento and Piemonte. In all cases diagnosed by culture examination, the etiological agent responsible for the disease was *L. pneumophila* [33-40].

<https://www.epicentro.iss.it/legionellosi/epidemiologia-italia> The prevention measures to be implemented are those appropriate to prevent the growth and development of the bacterium. In particular, all innovative and technologically advanced procedures and methods for cleaning and disinfecting the water system, any related device, cooling towers, evaporative towers, swimming pools and whirlpools must be carefully considered. In the field of health protection from infectious agents, it is necessary to consider, among the most important collective prevention-protection interventions, the disinfection activity that can be carried out through different methods, for example through manual procedures or using various types of equipment. Disinfection, which is very important for health protection, must be effective against all the biological agents that constitute the source of the infection or infections [41-60]. It is necessary to use disinfection methods that carefully consider the necessary contact times of the different substrates and possible interfering means, in which the infectious agents are present, since the microbicidal properties may be insufficient, cancelled out or greatly reduced. At the same time, it is necessary to be careful in the choice of formulations and compounds to be used, also evaluating the characteristics of toxicity for exposed subjects in relation to the concentrations of use.

Moreover, according to Italian legislation, in the case of disinfection of water distributed by the water system, it is important to avoid the formation of chemical compounds capable of causing exposure toxicity and/or making the water unsuitable for human consumption (Legislative Decree 31/2001 and subsequent amendments and additions (Legislative Decree 31/2001 and subsequent amendments and integrations. The choice of the disinfection method involves a careful priority selection of the compounds to be used also in the case of equipment that must use these active ingredients or formulations in order to comply with the safety measures established by the legislation in force. This legislation in Italy is Legislative Decree 81/2008 and subsequent amendments and additions (Consolidation Act on Health and Safety at Work - Implementation of Article 1 of Law no. 123 of 3 August 2007 on the protection of health and safety at work, transposition into Italian law of EU directives on health and safety at work) and in particular the provisions of Title X (transposition of Directive 54/2000 EC and subsequent amendments and additions) and Title I (transposition of Directive 391/89 EC and subsequent amendments and additions). In this regard, it is necessary to carefully consider the provisions of Article 15, paragraph 1, letter c) of the above-mentioned Title I, "elimination of risks in relation to knowledge acquired on the basis of technical progress and, where this is not possible, their reduction to a minimum", as well as the provisions of Art.

18 paragraph 1 letter z)" the employerupdates the measures of prevention that is, in relation to the degree of evolution of the technique of prevention and protection" and what is further highlighted by the jurisprudence of the sector [61-75]. In

choosing the disinfection methodology, it is important to examine the technical/scientific documentation in which, as in the case under observation, it can be established the effectiveness against microbial agents that, if present in the water of a water system, may be able to cause damage to the health of individuals who may be in a condition of risk of exposure. From this examination derives therefore the possibility of verifying compliance with the requirements of the aforementioned legislation (Legislative Decree 81/2008 and subsequent amendments and integrations).

Materials and Methods

A pilot plant has been designed and developed with a water circuit connecting to the chlorine dioxide generator mentioned above to verify the effectiveness of decontamination treatment from legionella and/or other pathogens. The study is carried out in the period 2017/ 2018. For the first experimental tests, September 2017 November 2017, both traditional methods, based on cultivation methods, and molecular tests related to the genetic analysis of bacterial DNA were used, using advanced techniques such as NGS (Next Generation Sequencing) applied to microflora DNA (mfDNA). The objective was to evaluate the disinfectant action of chlorine dioxide in an experimental pilot plant powered by the ClO₂ generator and the second experiment, carried out in May 2018, intended to verify the disinfectant action of Chlorine Dioxide on Legionella Specifically, these experimental tests were carried out using *Legionella pneumophila*, in order to verify the effectiveness of the disinfectant action of ClO₂ in the "water" array, and in particular considering its application within a water system. The pilot plant allows the tests to be conducted in such a way as to detect all chemical, physical, and microbiological parameters. The observed data therefore assume a particularly relevant meaning and can be attributed in a highly significant way to the exclusive action of the disinfection system used, demonstrating its effectiveness.

For the microbiological analysis, in addition to the evaluation of the total microbial charge, the experimental tests reported here, have specifically considered bacteria indicators of faecal contamination (*Escherichia coli*, *Enterococcus faecalis*): using the official procedures for the microbiological analysis of water and in particular ISTISAN protocols and / or based on defined substrate technology (Idexx), widely adopted and approved by Italian and international institutions [76-85]. For molecular analysis, amplification techniques have been used starting from rDNA and products tested for electrophoresis, real-time PCR or through NGS using Illumina system. Quality controls have been systematically carried out for both classical and molecular methods, confirming the validity of the cultivation tests and/or the absence of inhibitory agents on the amplification reaction. The microbial content was expressed in UFC (Colony Forming Units), MPN (Most Probable Number) or UG (Genomic Units), depending on the method reported. In the second experimentation's phase, both traditional methods, based on traditional cultivation methods (Guidelines for

the prevention and control of legionellosis', published in the Official Gazette of 5th May 2000; 'Guidelines on Legionellosis for managers of tourist accommodation and spa facilities' and 'Guidelines for laboratories with microbiological diagnosis and environmental control of legionellosis' (Official Gazette no. 28 of 4 February 2005 and Official Gazette no. 29 of 5 February 2005).

The guidelines have been updated in the light of new scientific knowledge, with the technical-scientific support of the Istituto Superiore di Sanità and institutional experts in the field.) by measuring the Colony Forming Units (CFU), and innovative microbiological tests based on defined substrate technology aimed at the definition of the Most Probable Number (MPN), as well as molecular tests based on genetic analysis of bacterial DNA measured in Genomic Units (UG) by Real Time PCR, were taken into consideration. Evaluations were performed at zero time (T₀), after inoculation from the moment of entry into circulation in the pilot plant (T_c) and after 5 minutes (T₅) and then at 30 minutes (T₃₀), in accordance with standard guidelines and protocols. Further tests at subsequent times were planned to verify the preservation of the disinfection efficacy over time even after repeated contamination, and in particular at 60 minutes (T₆₀) and 20 hours (T_{20H}). The experimental inoculations were performed by adapting standard protocols and UNI references, as previously described in the pilot unit for other experimental conditions, including tests on microbiological indicators, microflora assessments and other tests with Legionella simulating contamination conditions with high amounts of microorganisms and organic material. Samples were taken according to traditional procedures in the presence of sodium thiosulphate (Na₂S₂O₃).

Quality controls were systematically performed at least in duplicate for both classical and molecular methods, confirming the validity of the culture tests and/or the absence of inhibitory agents on the amplification reaction [86-95]. Simulating steady state conditions of use in a water system, two independent inoculations (>10⁹ CFU) were performed using ATCC strains of Legionella pneumophila on the pilot plant. The different tests were reproduced at least in duplicate. The chemical-physical parameters were collected using the specific test tubes included in the pilot unit. Microbiological measurements were expressed in UFCs (Colony Forming Units), MPNs (Most Probable Numbers) or UGs (Genomic Units), depending on the method used. The figure shows the data obtained by applying the current guidelines for the research of Legionella. Each evaluation was carried out in independent experiments and at least in duplicate for each of the expected sampling times ([Appendix 1](#)). These tests were performed on the pilot system through the introduction of particular improvements to control the different variables and monitor the main parameters in real time (online) during the experiment, including introduction of new sensors (eg. Chlorites, flow), modification of the recirculation and flow-rate systems with membrane pump, water heating with

stainless steel exchanger at controlled temperature, elimination of dead limbs and other causes of stagnation or turbulence in the flow, increase in the volume of the system (>50L), introduction of solutions to facilitate the picking and the inoculation (peristaltic pump and tank in series), in accordance with the needs of the experiment and safety for the operator.

In order to perform the experimental tests under stable and controlled conditions, the pilot plant had previously been brought to equilibrium and supervised continuously for one week in order to confirm the steady state for the different parameters, and to ensure the stability of the system and the different chemical-physical variables. Under these conditions the unit proved to be stable [96-105]. According to what was observed in the various tests before performing the inoculations, the variations were within the defined limits, and in particular: recirculation water temperature: >30 °C (maximum variation interval observed following injection/removal: 30-36 °C); chlorine dioxide: 0.2 ppm (maximum variation interval observed: 0.19-0.40); conductivity: 330-338 µS; chlorites: 0.230-0.707 µg/L (under storage conditions due to absence of

make-up and maintenance of the recirculation for 7 days in a total volume of about 50 Liters). The specific conditions reported at the time of inoculation with *Legionella pneumophila* were water temperature in the system: 36 and 34 °C; ClO₂: 0.241 and 0.251 ppm, in full agreement with what was previously observed.

Results

The tests carried out showed disinfectant efficacy on the various bacterial species already from the samples taken within 5 minutes (T₅) of exposition to the disinfectant (T₀), with a reduction near 100% (>99.999%) within 30 minutes. In particular:

- 1) *E. faecalis* showed a >99% reduction already within the first 5 minutes (Figures 2A & 2B).
- 2) *E. coli* showed a >99% reduction already within the first 5 minutes according to molecular data, even if in this experimental test the value could not be estimated as accurately in MPN, so it is prudentially reported the maximum value hypothesized. In any case, the reduction is confirmed >99% at 30' (Figures 3A & 3B).



Figure 2: Reduction of microbial load: *E. faecalis*.

Note: T₀: situation in the system at zero time; T_{contact}: analysis performed on sample taken immediately after inoculation; T₅: analysis performed on sample taken after 5 minutes; T₃₀: analysis performed on sample taken after 30 minutes.

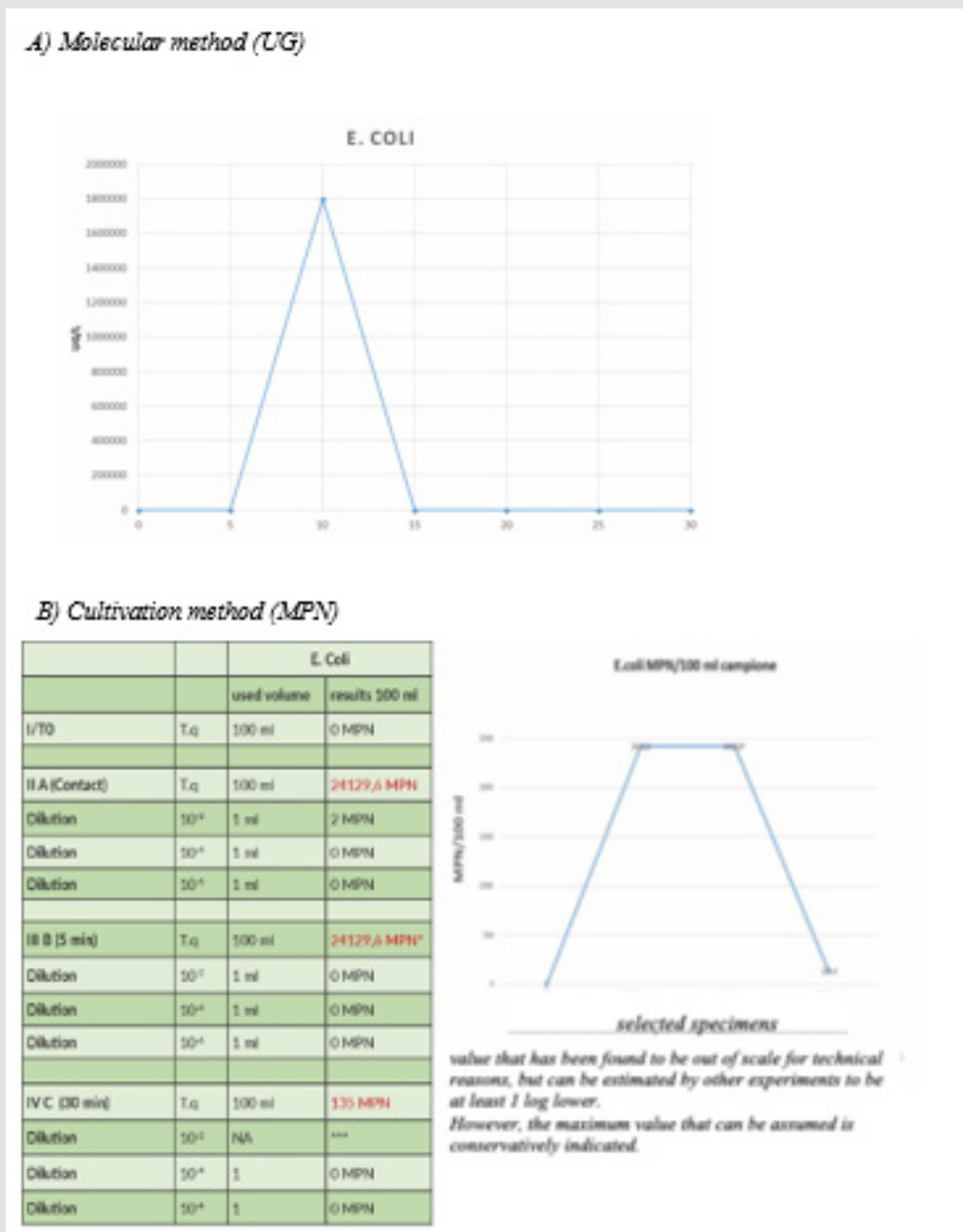


Figure 3: Reduction of microbial charge: *E. coli*.

Note: T₀: situation in the system at zero time; T_{contact}: analysis performed on sample taken immediately after inoculation; T₅: analysis performed on sample taken after 5 minutes; T₃₀: analysis performed on sample taken after 30 minutes. (NB: the data in UG on *E. Coli* refer to a validity threshold < 33 CT as the tests have been performed using recombinant Taq. In addition, the MPN value at T₅ is overestimated).

3) The total microbial charge, including the biofilm component in the supply water and in the system, is reduced by more than 99% following the activation of the disinfectant equipment able to produce Chlorine Dioxide. This observation is demonstrated both by the cultivation tests (Figure 4A) and by a molecular test which is able to significantly identify both the indicators used and the complexity of the bacterial microflora

present in its different bacterial species (Figure 4B). In addition, the integration (Figure 4A) of a further experiment carried out over a longer period (2-3 months) and aimed at verifying the abatement capacity of the microflora present in the water inlet of the system is also reported. The disinfectant action appears effective and active on different bacterial species.



Figure 4.

In addition, the 2018 trial showed disinfectant efficacy on *Legionella pneumophila*, with a killing rate close to 100% (>99.999%) within 30 minutes. Even after repeated contamination, the system proved to be able to demonstrate the maintenance of disinfectant efficacy over time, even after 1 hour and 20 hours after subsequent inoculations of the order of billions of viable bacterial cells. The pilot plant had previously been balanced and the various parameters kept under control for at least one week before the contamination experiment was carried out. The temperature of the implant was maintained at conditions above 30 °C to promote the survival of *Legionella* by performing the contaminations at 36 and 34 °C. During the experiment, the values of Chlorine Dioxide were observed to be kept within the prescribed limits, i.e. in particular between 0,242 and 0,251 ppm. The values reported for Chlorites also remained within acceptable limits and in any case always < 0,7

µg/L, even in conditions of prolonged accumulation in the absence of dilution for reintegration (Appendix 2). The killing of *Legionella pneumophila* was demonstrated not only by using the traditional procedures provided for in the guidelines, but also in parallel with innovative methods based on defined substrate technology, fully confirming the disinfectant efficacy of ClO₂, and in particular the complete elimination of viable cells of *Legionella* within 30' of inoculation.

The presence of bacteria in the implant was also evaluated by molecular methods, further validating the microbial load, and demonstrating the presence of dead bacterial cells following the treatment in question (>108UG/L) (Figure 5). All these observations allow sustaining with high relevance the disinfectant efficacy on *Legionella pneumophila* of the Chlorine Dioxide released inside the pilot unit in question.

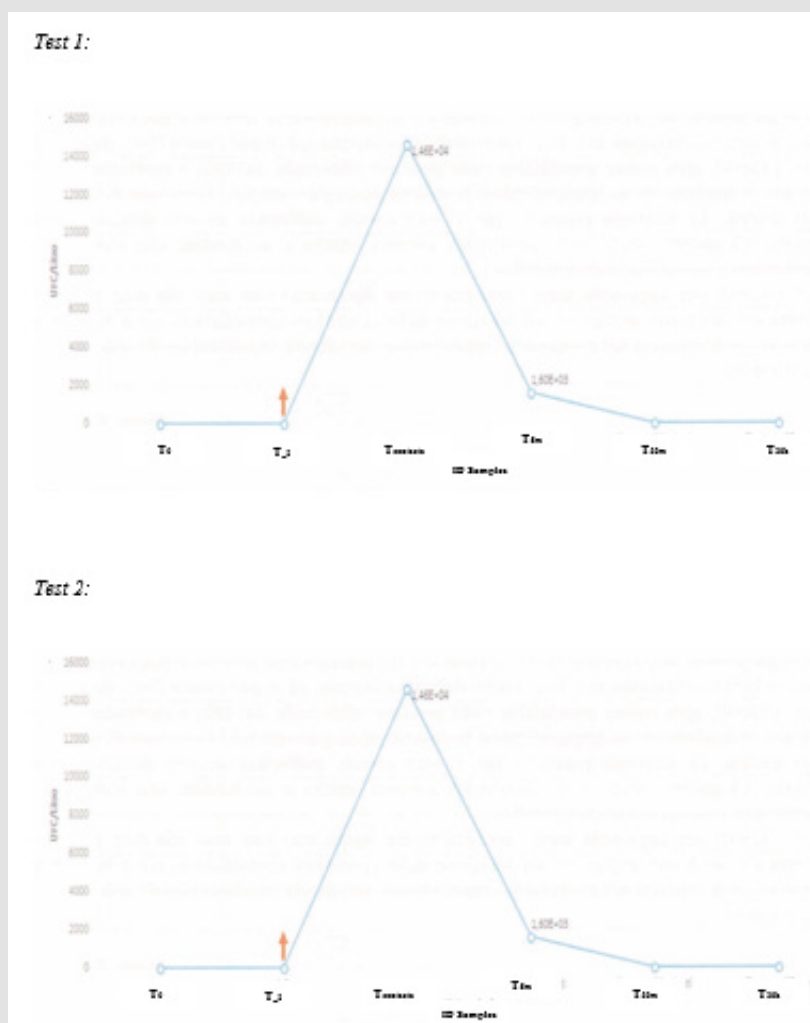


Figure 5: Reduction of microbial load: *Legionella pneumophila*.

Note: T₀: situation in the system at zero time; T_{contact}: analysis performed on sample taken immediately after inoculation; T₅: analysis performed on sample taken after 5 minutes; T₃₀: analysis performed on sample taken after 30 minutes.

Discussion

The disinfection treatment in question proved effective in killing a Gram-positive bacterium (*E. faecalis*) and a Gram-negative bacterium (*E. coli*), both indicators of faecal contamination. However, the disinfectant action is also evident on the total bacterial count as it can act on several other microbial species, as shown by the application of both traditional and molecular cultivation methods based on bacterial DNA analysis. The indication of disinfectant efficacy, even on a wider range of environmental species present in aquatic microflora or biofilm, suggests the applicability of this treatment also for the clean-up of installations or for the processing of supply waters that do not fully satisfy the essential requirements of potability. Among these micro-organisms that can be found in water systems there may be some pathogens, including enterobacteria, or species belonging to the family of Legionellaceae. The pilot plant designed and developed could be a model for further

investigations, especially in the light of the experimental protocols adapted for exposition, sampling, analysis, even in the presence of multiple contaminations [106-114]. The treatment system based on the action of the chlorine dioxide generator in the study shows an effective disinfectant action. The results reported are particularly significant, therefore, not only considering the observations acquired, but also considering the controlled conditions in which it was possible to perform the tests of effectiveness of the disinfectant treatment, simulating conditions verifiable within a water system.

The disinfection intervention has been more effective in killing *Legionella pneumophila*. This observation is in addition to the previous ones, allowing to acquire information strongly significant in terms of disinfectant effectiveness even after particularly high contamination (>109 UFC) and supporting a clear disinfectant action also for other microbial species and, more generally, for the reduction of the total bacterial charge. It is important to note that,

although some initial experimental conditions could adversely affect 1 the reported values, Chloritis remained within acceptable limits. In the light of the various information acquired, the treatment system based on the action of Chlorine Dioxide from the generator, used in the pilot plant, shows an effective disinfectant action against Legionella and a particularly valid approach to use because it is based on real-time monitoring of various parameters through an integrated and automated management philosophy. Note, as a further remarkable aspect, that also the results reported for Chlorites remained within the acceptable limits, although the starting limits at the time of the experimental inoculation trials of the bacterium were already relatively high due to the introduction of ClO₂, kept at steady state for more than a week in the absence of reintegration of the introduction of disinfectant automatically and also following the inoculation with organic material, the volume in scale of the installation and the inevitable accumulation due to the closed system of recirculation of the installation in size ([Appendix 3](#)).

In consideration of the different information acquired, the treatment system based on the action of Chlorine Dioxide and studied through the pilot unit previous described, therefore, shows an effective disinfectant action against Legionella and a particularly valid approach of use, as it is based on the real-time monitoring of various parameters through an integrated and atomized management of the disinfection system. For applications on extended installations of different types, not only in terms of safety but also quality, the proposed water treatment policy is therefore effective and promising. The pilot installation has proved to be a stable and modular system that merits further study and technical-scientific development. The results reported for Legionella are particularly significant not only in the context of the various observations collected, but also in consideration of the controlled conditions in which it was possible to perform tests on the effectiveness of the disinfectant treatment, simulating conditions that could be verified inside a water system.

In conclusion, regarding the efficacy profile of the analyzed ClO₂ generator, an interesting decontamination activity has been observed even in the case of water, which for various reasons does not present suitable characteristics of potability and an important and more than significant possible decontamination activity against different microbial agents, such as yeasts/fungi and bacteria, among the latest we can indicate as frequent contaminants *Pseudomonas aeruginosa*, *E. coli*, Legionellaceae.

A more than relevant and significant activity has been demonstrated towards Legionella pneumophila under measurable conditions of stressed system, through analytical determinations, also of an extremely innovative and technologically advanced type, allowing the extrapolation of the results obtained also for other identical equipment but with superior functional capacities. From a careful examination of the experimental results and of the technical-scientific literature on the subject and, considering the reference

legislation of the sector already mentioned, the treatment of disinfection of the water system that uses the mentioned generator of ClO₂ qualifies among the safety measures to be implemented for the risk of infectious agents and is considered to comply with the provisions of the current Italian and Community legislation, i.e. Legislative Decree 81/2008 and subsequent amendments and integrations, (transposition of EU Directives on hygiene and safety at work), with particular reference to Title X (transposition of Directive 54/2000 EC) and Title I (transposition of Directive 391/89 EC).

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